

N (25, 26, 34, 35, and 38) = 82% C; 6% G, T, A

N (19, 22, and 37) = 82% G; 6% C, T, A

N (24, 27, 30, 33, and 36) = 82% T; 6% G, C, A. Numbers in parentheses correspond to the positions above in SEQ ID NO:18, wherein the first G is position number 1.

The mutagenic primer D corrects the frame-shift mutation and eliminates the *Bam*HI and *Bln*I sites introduced into pEG359. To accomplish this mutagenesis, the Megaprimer was first synthesized by PCR™ amplification of pEG315 DNA (FIG. 2) using the mutagenic primer D and the opposing primer C (FIG. 5). The resulting amplified DNA fragment was purified by gel electrophoresis as described above and used in a second PCR™ using primers A and C and p154 as the template. Because the p154 template contains a deletion of the region complementary to primer C (FIG. 5), initiation of the PCR™ first requires extension of the Megaprimer to allow annealing of primer A to the mutagenic strand, thus ensuring that most of the amplified product obtain from the PCR™ incorporates the mutagenic DNA. The resulting PCR™ product was isolated and purified following gel electrophoresis in agarose and 1X TAE as described above.

The amplified DNA fragment was digested with the restriction enzymes *Age*I and *Bbu*II, to provide sticky ends suitable for cloning, and with the enzymes *Bam*HI and *Bln*I to eliminate any residual p154 template DNA. pEG359 was digested with *Age*I and *Bbu*II and the vector fragment ligated to the restricted amplified DNA preparation. The ligation reaction was used to transform the *E. coli* Sure™ (Stratagene Cloning Systems, La Jolla, CA) strain to ampicillin (Amp) resistance (Amp<sup>R</sup>) using a standard transformation procedure. Amp<sup>R</sup> colonies were scraped from plates and growth for 1-2 hr at 37°C in Luria Broth with 50 µg/ml of Amp. Plasmid DNA was isolated from this culture using the alkaline lysis procedure described above and used to transform *B. thuringiensis* EG10368 to Cml resistance (Cml<sup>R</sup>) by electroporation. Transformants were plated on starch agar plates containing 5 µg/ml Cml and incubated at 25-30°C. Restriction enzyme analysis of plasmid DNAs isolated from crystal-forming transformants indicated that ~75% of the transformants had incorporated the mutagenic oligonucleotide at the target

site (nt 352-372). That is, ~75% of the crystal-forming transformants had lost the *Bam*HI and *Bln*I sites at the target site on *cry*IC.

### 5.3 EXAMPLE 3 -- MUTAGENESIS OF ARG RESIDUES IN CRY1C DOMAIN 1

Arginine residues within potential loop regions of Cry1C domain 1 were replaced by alanine residues using oligonucleotide-directed mutagenesis. The elimination of these arginine residues may reduce the proteolysis of toxin protein by trypsin-like proteases in the lepidopteran midgut since trypsin is known to cleave peptide bonds immediately C-terminal to arginine and lysine. The arginine residues at amino acid positions 148 and 180 in the Cry1C amino acid sequence were replaced with alanine residues. The PCR<sup>TM</sup>-mediated mutagenesis protocol used, described by Michael (1994) relies on the use of a thermostable ligase to incorporate a phosphorylated mutagenic oligonucleotide into an amplified DNA fragment. The mutagenesis of R148 employed the mutagenic primer E (SEQ ID NO:19) and the flanking primers A (SEQ ID NO:15) and primer F (SEQ ID NO:20). The mutagenesis of R180 employed the mutagenic primer G (SEQ ID NO:21) and the flanking primers A (SEQ ID NO:15) and F (SEQ ID NO:20). Both PCR<sup>TM</sup> studies employed pEG315 (FIG. 2) DNA as the *cry*IC template. Primer E was designed to eliminate an *Asu*II site within the wild-type *cry*IC nucleotide sequence. Primer G was designed to introduce a *Hinc*II site within the *cry*IC nucleotide sequence.

Primer E: (SEQ ID NO:19)

5'-GGGCTACTTGAAAGGGACATTCCTTCGTTTGAATTTCTGGATTGAAGTACCCC-3'

Primer F: (SEQ ID NO:20)

5'-CCAAGAAAATACTAGAGCTCTTGTTAAAAAAGGTGTTCC-3'

Primer G: (SEQ ID NO:21)

5'-GAGATTCTGTAATTTTTGGAGAAGCATGGGGGTTGACAACGATAAATGTC-3'

The products obtained from the PCR<sup>TM</sup> were purified following agarose gel electrophoresis using the GeneClean II® procedure and reamplified using the opposing primers A and F and standard PCR<sup>TM</sup> procedures. The resultant PCR<sup>TM</sup> products were digested with the restriction enzymes *Bbu*I and *Age*I. pEG315, containing the intact *cry*IC gene of EG6346, was digested with the restriction enzymes *Bbu*I and *Age*I. The

restricted fragments were resolved by agarose gel electrophoresis in 1X TAE, the pEG315 vector fragment purified using the GeneClean II® procedure and, subsequently ligated to the amplified DNA fragments obtained from the mutagenesis using T4 ligase. The ligation reactions were used to transform the *E. coli* DH5α™ to Amp resistance using standard transformation methods. Transformants were selected on Luria plates containing 50 µg/ml Amp. Plasmid DNAs isolated from the *E. coli* transformants generated by the R148 mutagenesis were used to transform *B. thuringiensis* EG10368 to Cml<sup>R</sup>, using the electroporation procedure described by Mettus and Macaluso (1990). Transformants were selected on Luria plates containing 3 µg/ml Cml. Approximately 75% of the EG10368 transformants generated by the R148 mutagenesis had lost the *Asu*II site, indicating that the mutagenic oligonucleotide primer E had been incorporated into the *cry*IC gene. One transformant, designated EG11811, was chosen for further study. Approximately 25% of the *E. coli* transformants generated by the R180 mutagenesis contained the new *Hinc*II site introduced by the mutagenic oligonucleotide primer G, indicating that the mutagenic oligonucleotide had been incorporated into the *cry*IC gene. Plasmid DNA from one such transformant was used to transform the *B. thuringiensis* host strain EG10368 to Cml<sup>R</sup> by electroporation as before. One of the resulting transformants was designated EG11815.

The mutagenesis of R148 was repeated using the *cry*IC gene contained in plasmid pEG345. Plasmid pEG345 (FIG. 2) contains the *cry*IC gene from *B. thuringiensis* subsp. *aizawai* strain 7.29 (Sanchis *et al.*, 1989; Eur. Pat. Application EP 295156A1; Intl. Pat. Appl. Publ. No. WO 88/09812). The mutagenesis of R148 employed the mutagenic primer E (SEQ ID No: 19), the flanking primers H (SEQ ID NO:52) and F (SEQ ID NO:20), and plasmid pEG345 as the source of the *cry*IC DNA template. Primer E was designed to eliminate an *Asu*II site within the wild-type *cry*IC sequence.

Primer H: 5'-GGATCCCTCGAGCTGCAGGAGC-3' (SEQ ID NO:52)

*cry*IC template DNA was obtained from a PCR™ using the opposing primers H and F and plasmid pEG345 as a template. This DNA was then used as the template for a PCR™-mediated mutagenesis reaction that employed the flanking primers H and F and the mutagenic oligonucleotide E, using the procedure described by Michael (1994). The